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13. SUPPLEMENTARY NOTES					
14. We have discovered RNA splicing as a novel mechanism underlying tumor aggressiveness and drug resistance in African American (AA) prostate cancer (PCa). To interrogate further the contribution of RNA splicing to the more aggressive PCa biology in AA men, we are collecting AA and white PCa patient blood and tissue specimens of varying Gleason grade for study. In addition, we have identified RNA splicing regulatory variants that associate with PCa risk, aggressiveness and/or survival. Furthermore, we have developed a splice-switching oligonucleotide (SSO) that inhibits production of a pathogenic androgen receptor (AR) variant at the RNA- and protein-level, while maintaining expression of full-length AR, which has therapeutic value. This SSO inhibits proliferation of PCa cells and restores sensitivity to an AR inhibitor. In addition, we have developed SSOs that drive production of inhibitory dominant-negative epidermal growth factor receptor (EGFR) isoforms at the RNA-level and decrease phosphorylated EGFR protein. Ultimately, this study will establish novel biological differences between AA and white PCa and their relevance to tumor biology, which will aid in the development of new biomarkers and/or therapeutics that will reduce PCa health disparities for AAs and improve outcomes for men of all races with aggressive disease.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	9
5. Changes/Problems.....	10
6.Products.....	10
7. Participants & Other Collaborating Organizations.....	12
8. Special Reporting Requirements.....	14
9. Appendices.....	N/A

All pages contain proprietary/unpublished data.

INTRODUCTION: African American (AA) men exhibit 2-fold higher incidence and 3-fold higher mortality rates from prostate cancer compared to white men. Although much of this disparity remains after controlling for factors related to access to care, very few studies have utilized this population-based difference to identify molecular mechanisms of tumor aggressiveness. The studies proposed here address the urgent need to elucidate the molecular mechanisms underlying the more aggressive prostate cancer biology in AA men. Our objectives are to 1) expand our sample cohort and delineate the relationship between genetic/epigenetic/post-transcriptional factors in AA prostate cancer and Gleason grade and 2) manipulate splicing using novel splice-switching oligonucleotides and determine functional outcomes. Establishing the underlying genetic/epigenetic/post-transcriptional differences between AA and white prostate cancer and the biologic relevance of these differences to tumor biology will identify novel precision biomarkers and/or molecular targets for precision medicine interventions that will have profound implications for the prevention, screening, diagnosis and management of prostate cancer in AA men as well as men of all races with aggressive disease. Specifically, if positive, these genetic/epigenetic/post-transcriptional differences could be developed as prognostic markers, in the context of Gleason grade and other prognostic variables, to delineate patients at greater risk of progressing on active surveillance or through localized therapy. In addition, the causal relationship of these pathways would help to rationalize specifically targeted therapy in selected patients.

KEYWORDS (20 words): Prostate cancer, health disparities among racial groups, molecular mechanisms, differential gene expression, alternative RNA splicing, epigenetic alterations, clinical tumor aggressiveness

ACCOMPLISHMENTS:

What were the major goals of the project?

- Task 1. Validate differentially expressed and spliced candidate genes in AA prostate cancer in an expanded sample cohort and define the relationship between these genes and Gleason grade. Months 1-21. 75% complete (please see progress for Task 1).
- Task 2. Define the biologic significance of differences in *cis*-acting splicing elements of alternatively spliced candidate genes in AA prostate cancer to alternative splicing events specific to AA prostate cancer and define the relationship between these patterns and Gleason grade. Months 21-36. 50% complete (please see data for Task 2).
- Task 3. Use splice-switching oligonucleotides to delineate the biologic relevance of race-related differentially spliced genes involved in PIK3CD and MET signaling. Months 1-24. 75% complete (please see data for Task 3).

What was accomplished under these goals?

Progress for Task 1: We continue to obtain individual patient AA and white prostate cancer tissue specimens and patient-matched blood specimens under our IRB approved GENomics of CANcer DisparitiEs (GENCADE) protocol and capture annotated data in our accompanying database. For all tissue specimens collected to date, we have screened the specimens for tumor content, determined the Gleason grade, isolated cellular DNA and RNA, confirmed adequacy of yield and quality of these nucleic acids for downstream analyses and annotated the specimens. For white prostate cancer specimens, we have completed our collection of Gleason 7 and Gleason 8-10 specimens, and need to collect 3 additional Gleason 6 specimens to fully complete collection of all groups for downstream analyses. For AA prostate cancer specimens, 6 additional Gleason 6 specimens, 4 additional Gleason 7 specimens and 9 additional Gleason 8-10 specimens remain to be collected to fully complete collection of all groups for downstream analyses. Currently, we have prostate cancer specimens collected from 8 white and 17 AA patients that are pending eligibility verification (tumor content, Gleason grade and DNA/RNA yield and quality). We obtained a one year no cost extension and plan to complete collection of the aforementioned specimens within the next 6 months. We will then complete downstream analyses by the end of the year.

Data for Task 2: While we continue to obtain individual patient AA and white prostate cancer tissue specimens and patient-matched blood specimens, we have completed three additional analyses regarding the clinical significance of differences in *cis*-acting splicing elements of alternatively spliced genes in AA and white prostate cancer.

The citation for the first study is Wang Y*, J.A. Freedman*, X. Li, P.G. Moorman, D.J. George, T. Hyslop, S.R. Patierno** and Q. Wei**. 2017. Associations between RNA splicing regulatory variants of stemness-related genes and racial disparities in susceptibility to prostate cancer. *International Journal of Cancer* 141(4): 731-743. [*Contributed equally as co-first authors, ** Contributed equally as co-PIs] PMID: 28510291. The abstract for this study follows. Evidence suggests that cells with a stemness phenotype play a pivotal role in oncogenesis, and prostate cells exhibiting this phenotype have been identified. We used two genome-wide association study (GWAS) datasets of African descendants, from the Multiethnic/Minority Cohort Study of Diet and Cancer (MEC) and the Ghana Prostate Study, and two GWAS datasets of non-Hispanic whites, from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial and the Breast and Prostate Cancer Cohort Consortium (BPC3), to analyze the associations between genetic variants of stemness-related genes and racial disparities in susceptibility to prostate cancer. We evaluated associations of single-nucleotide polymorphisms (SNPs) in 25 stemness-related genes with prostate cancer risk in 1,609 cases and 2,550 controls of non-Hispanic whites (4,934 SNPs) and 1,144 cases and 1,116 controls of African descendants (5,448 SNPs) with correction by false discovery rate ≤ 0.2 . We identified 32 SNPs in five genes (TP63, ALDH1A1, WNT1, MET and EGFR) that were significantly associated with prostate cancer risk, of which six SNPs in three genes (TP63, ALDH1A1 and WNT1) and eight EGFR SNPs showed heterogeneity in susceptibility between these two racial groups. In addition, 13 SNPs in MET and one in ALDH1A1 were found only in African descendants. The in silico bioinformatics analyses revealed that EGFR rs2072454 and SNPs in linkage with the identified SNPs in MET and ALDH1A1 ($r^2 > 0.6$) were predicted to regulate RNA splicing. These variants may serve as novel biomarkers for racial disparities in prostate cancer risk.

A manuscript reporting our findings from our second study is in preparation. Its citation will be Freedman JA*, Wang Y*, Li X, Moorman PG, George DJ, Hyslop T, Wei Q**, Patierno SR**, Single nucleotide polymorphisms of stemness pathway genes predicted to regulate RNA splicing, microRNA and oncogenic signaling are associated with prostate cancer survival, *Contributed equally as co-first authors, **Contributed equally as Co-PI's. We will submit this manuscript to *Cancer Epidemiology, Biomarkers & Prevention*. The abstract for this study follows and please see ***Figure 1-3*** and ***Table 1-3*** at the end of this progress report for a summary of our most important findings. Prostate cancer is a clinically and molecularly heterogeneous disease, with variation in outcomes only partially predicted by grade and stage. Additional tools to distinguish indolent from aggressive disease are needed. Phenotypic characteristics of stemness correlate with poor cancer prognosis. Given this correlation, we identified single nucleotide polymorphisms (SNPs) of stemness pathway genes and examined their associations with prostate cancer survival. SNPs within stemness pathway genes were analyzed for association with overall survival of prostate cancer in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. Significant SNPs predicted to be functional were selected for linkage disequilibrium analysis and combined and stratified analyses. Validated SNPs were evaluated for association with gene expression. SNPs of *CD44* (rs9666607), *ABCC1* (rs35605 and rs212091) and *GDF15* (rs1058587) were associated with prostate cancer survival and predicted to be functional. A role for rs9666607 of *CD44* and rs35605 of *ABCC1* in RNA splicing regulation, rs212091 of *ABCC1* in miRNA binding site activity and rs1058587 of *GDF15* in causing an amino acid change was predicted. These SNPs represent potential novel prognostic markers for overall survival of prostate cancer and support a contribution of the stemness pathway to prostate cancer patient outcome.

In the third study, as shown in ***Figure 4*** at the end of this progress report, we have explored associations between genetic variants of 30 of the alternatively spliced genes in AA and white prostate cancer and prostate cancer risk, aggressiveness and/or survival in white and AA groups by analyzing published genome-wide association studies (GWAS) of prostate cancer. Specifically, we used GWAS datasets from the Multiethnic Cohort Study of Diet and Cancer (MEC), including AA prostate cancer cases and controls, and the Prostate,

Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO), including white prostate cancer cases and controls, to evaluate associations of 11,073 and 10,385 SNPs, respectively, in 30 genes identified to be alternatively spliced between white and AA prostate cancer with prostate cancer risk, aggressiveness and/or survival. For risk, we evaluated 1150 cases and 1101 controls in PLCO and 670 cases and 658 controls in MEC, for aggressiveness, we evaluated 237 aggressive and 843 non-aggressive in PLCO and 234 aggressive and 436 non-aggressive in MEC, and for survival, we evaluated 1150 overall, 237 aggressive and 843 non-aggressive in PLCO. We then performed *in silico* bioinformatics to investigate potential functions of the SNPs. Significant associations between SNPs in *FNI*, *COL6A3* and *ACACA* and SNPs in *SEMA3C* and *FASN* and PC risk in white and AA populations, respectively, were identified. In addition, SNPs in *ACACA* and SNPs in *SEMA3C*, *RELN*, *MYBPC1*, *NCOR2* and *WDR4* were found to be significantly associated with prostate cancer aggressiveness in white and AA populations, respectively. Furthermore, significant associations between SNPs in *RHOU*, *FNI*, *COL6A3*, *SEMA3C*, *RELN*, *CD44*, *LMO7* and *WDR4* and prostate cancer survival in a white population were identified. All of the aforementioned SNPs were predicted to play a role in splicing regulation. In conclusion, SNPs of race-related alternatively spliced genes that are predicted to play a role in splicing regulation are significantly associated with prostate cancer risk, aggressiveness and/or survival in white and/or AA populations. Such variants have the potential to serve as novel molecular targets for development of biomarkers of increased risk of aggressive prostate cancer or therapeutics against aggressive prostate cancer. Ultimately, such biomarkers and therapeutic agents could serve as novel precision medicine interventions, reducing the mortality burden from prostate cancer among AA men.

Data for Task 3: Previous work from our laboratory and others have identified deregulation of the androgen receptor (AR) and epidermal growth factor receptor (EGFR) pathways in AA versus white prostate cancer. Thus, there is an urgent need to develop a novel therapeutic strategy capable of inhibiting oncogenic AR and EGFR RNA isoforms enriched for in AA prostate cancer. We have further assessed the functional effects of our novel chemically modified splice switching oligonucleotide (SSO) targeting androgen receptor variant 7 (AR-V7). We designed this SSO to inhibit splicing leading to production of AR-V7, a pathogenic AR variant that lacks the ligand binding domain, is constitutively active and associates with prostate cancer progression and resistance to androgen ablation/AR inhibition therapies, while simultaneously maintaining splicing leading to production of full-length AR, which has therapeutic value (sensitivity to androgen ablation/AR inhibition therapies). As shown in [Figure 5](#) at the end of this progress report, transfection of a panel of white and AA prostate cancer cell lines with the AR-V7 SSO inhibits AR-V7 RNA and protein in a dose dependent manner compared to transfection with a control scrambled oligonucleotide. In the same cells, post-transfection, we observe maintenance of full-length AR expression at the RNA- and protein-level. Furthermore, this biochemical response associates with a biologically significant phenotype, as we also observe inhibition of prostate cancer cell proliferation post-transfection with the AR-V7 SSO compared to a control scrambled oligonucleotide. Finally, we also observe that inhibition of AR-V7 and maintenance of full-length AR associates with restoration of sensitivity to enzalutamide, an AR inhibition therapy, as we observe inhibition of prostate cancer cell proliferation in the presence of enzalutamide post-transfection with the AR-V7 SSO compared to a control scrambled oligonucleotide.

In addition, as shown in [Figure 6](#) at the end of this progress report, we have also designed SSOs to drive production of inhibitory dominant-negative EGFR isoforms (EGFR-TM or EGFR-TK SSOs), lacking the transmembrane or tyrosine kinase domain, respectively. Transfection of prostate cancer cell lines with EGFR-TM or EGFR-TK SSOs increase RNA corresponding to these isoforms in a dose-dependent manner. Furthermore, a decrease in phosphorylated EGFR protein is detected.

Along with our qualified collaborator and his laboratory, we published our discovery of alternative RNA splicing as a novel molecular mechanism underlying tumor aggressiveness and drug resistance in AA prostate cancer. The citation for this study is Wang BD, Ceniccola K, Hwang S, Andrawis R, Horvath, A, Freedman JA, Olender, J, Knapp S, Ching T, Garmire L, Patel V, Garcia-Blanco, MA, Patierno SR, Lee NH. Alternative splicing promotes tumour aggressiveness and drug resistance in African American prostate cancer. *Nature Communications*. 2017 Jun 30;8:15921. PMID 28665395. The abstract for this study follows. Clinical

challenges exist in reducing prostate cancer (PCa) disparities. The RNA splicing landscape of PCa across racial populations has not been fully explored as a potential molecular mechanism contributing to race-related tumour aggressiveness. Here, we identify novel genome-wide, race-specific RNA splicing events as critical drivers of PCa aggressiveness and therapeutic resistance in African American (AA) men. AA-enriched splice variants of *PIK3CD*, *FGFR3*, *TSC2* and *RASGRP2* contribute to greater oncogenic potential compared with corresponding European American (EA)-expressing variants. Ectopic overexpression of the newly cloned AA-enriched variant, *PIK3CD-S*, in EA PCa cell lines enhances AKT/mTOR signalling and increases proliferative and invasive capacity in vitro and confers resistance to selective PI3K δ inhibitor, CAL-101 (idelalisib), in mouse xenograft models. High *PIK3CD-S* expression in PCa specimens associates with poor survival. These results highlight the potential of RNA splice variants to serve as novel biomarkers and molecular targets for developmental therapeutics in aggressive PCa.

Now that we have successfully completed our analyses of alternative splicing of *PIK3CD* in white versus AA prostate cancer, we have designed and synthesized chemically modified SSOs to manipulate the alternative splicing of this target. One of the challenges of developing SSOs involves identification of the sequences that block the desired splice sites most efficiently. Therefore, we have designed and synthesized additional oligonucleotides consisting of sequences that span various regions of the splice sites we are interested in targeting plus 4 nucleotides upstream and downstream of these sequences to generate SSOs that block the splice sites most efficiently. We are in the process of testing the effects of these SSOs on *PIK3CD* splicing and prostate cancer cell biology.

In parallel to the work being done in the context of this grant, we have established a number of AA and white prostate cancer patient-derived primary cell lines and xenografts by transferring primary human tumors directly from patients onto fibroblast feeder cells or into immunodeficient mice, respectively. These models provide invaluable tools, generated from racially diverse patients and preserving histology patterns from their human counterparts. Using such patient-derived models, we expect to be able to apply results to the clinical setting. The importance of this cannot be understated: our work represents the first significant collection of AA prostate cancer patient-derived primary cell lines and xenografts. Please see [Figure 7](#) at the end of this progress report for representative examples. We will assess the therapeutic efficacy of our aforementioned SSOs in these AA and white prostate cancer patient-derived primary cell lines and xenograft models.

What opportunities for training and professional development has the project provided?

This project provided training and professional development for Jennifer A. Freedman, PhD, Co-investigator and Bonnie LaCroix, Lab Research Analyst II. Dr. Freedman has expanded her expertise in performing all aspects of translational research by writing the GENCADE IRB protocol, designing the GENCADE REDCaP database, creating the GENCADE IRB-approved patient handout and collaborating with members of the Genitourinary Oncology clinical research team to implement the GENCADE Study. In addition, this project gave her an opportunity to collaborate with molecular epidemiologists to identify variants of stemness-related genes predicted to regulate RNA splicing that associate with racial disparities in susceptibility to prostate cancer. This team also identified SNPs of stemness pathway genes predicted to regulate RNA splicing, microRNA and oncogenic signaling that associate with prostate cancer survival. Moreover, Dr. Freedman has gained additional expertise in the design of SSOs and assessment of their functional effects on splicing and prostate cancer cell biology. Furthermore, she continues to increase her knowledge regarding development of AA and white prostate cancer models and prostate cancer health disparities among racial groups. Finally, this project has provided an opportunity for Dr. Freedman to develop further her skills in scientific management and mentoring of members of the Genitourinary Oncology Laboratory, including the Lab Research Analyst II working on this project. Dr. Freedman and Mrs. LaCroix attended and presented findings from this project at the AACR Cancer Health Disparities Conference last month, with Dr. Freedman giving an oral presentation in the Hot Topics in Cancer Health Disparities 1 section and Mrs. LaCroix giving the corresponding poster presentation. Dr. Freedman recently received and accepted an invitation to give a platform presentation during the Population Specific Research on Cancer Targeted Treatments and Drug Response: Contributing to the Elimination of Cancer Health Disparities session of the AACR Annual Meeting 2018. Mrs. LaCroix has

expanded her technical molecular biology expertise in assessing the effects of SSOs on splicing and prostate cancer cell biology and her knowledge of prostate cancer health disparities among racial groups.

Our Genitourinary Oncology Laboratory also has two postdoctoral associates who are working on parallel, but related projects and have benefited from exposure to the research conducted in the context of this grant. Dr. April Deveau is an African American scientist who conducted her PhD thesis research on genetic/biological aspects of prostate cancer disparities at Howard University Medical Center in Washington DC. She recently completed medical school at the University of North Carolina, Chapel Hill, and has elected to return to laboratory research for the next phase of her training. Since joining our laboratory, Dr. Deveau has expanded her repertoire of technical molecular biology skills, furthered her knowledge of cancer health disparities and honed her presentation and writing skills. Last year she gave a podium presentation at our Duke Cancer Institute Scientific Retreat and this year she received an AACR Scholar-in-Training Award to the AACR Cancer Health Disparities Conference. Dr. Muthana Al Abo is a Syrian scientist who conducted his PhD thesis research and initial postdoctoral research on molecular mechanisms of DNA repair and anti-cancer agents targeting this pathway. Since recently joining our laboratory, Dr. Al Abo has gained knowledge of cancer health disparities and is applying his technical molecular biology and computational skills to this area. He submitted an abstract on some of his initial findings to the upcoming AACR Special Conference on Prostate Cancer.

Finally, Mr. Brendon Patierno, a Research Technician II in our laboratory is generating the AA and white prostate cancer patient-derived primary cell lines and xenografts. Since joining our laboratory, he has gained these skills and knowledge of cancer health disparities, including the urgent need to generate such models. Because of this positive experience, Mr. Patierno is now pursuing application to graduate school in biology.

How were the results disseminated to communities of interest?

We have undertaken outreach activities to increase participation, including minority participation, in our GENCADE Study. In collaboration with the Duke Cancer Institute's Office of Health Equity and Disparities (OHED), we have approached patients participating in our annual community Men's Health Fair and have designed, produced and implemented use of our GENCADE IRB-approved patient handout. We have also worked with OHED to design and implement a training program called JUST ASK, for all staff participants in GENCADE and other clinical trials, centered on culturally sensitive and competent communications with patients about clinical trials.

In addition, we have broadly disseminated our vision and results through a number of mechanisms. As mentioned above, OHED is engaged in community outreach and programming, including an annual Men's Health Fair in inner city Durham North Carolina (38% African American), patient navigation and minority accrual to clinical trials. Through these efforts, we shared our vision and results with the public, patients and community practitioners. In addition, we are part of Duke Cancer Institute's Genitourinary Oncology Program, directed by Dr. George. This Program includes translational scientists and clinical investigators from Medical Oncology, Urology and Radiation Oncology. The Program sees prostate cancer patients of all races and ethnicities, places patients onto clinical trials and, as a member of the Department of Defense Prostate Cancer Clinical Trial Consortium, contributes to investigation of novel agents. Thus, we have discussed our vision and results with patients and practitioners through forums, tumor boards and grand rounds. Dr. Patierno, PI of this grant and Deputy Director of the Duke Cancer Institute, Dr. George, as Director of the Genitourinary Oncology Program and additional Duke Cancer Institute leaders regularly reach out to investors and donors. Through these events, we shared our vision and results with such parties. Finally, via our relationships with the Peter Michael Foundation, Prostate Cancer Foundation, American Cancer Society, American Association for Cancer Research and American Society of Clinical Oncology, Duke Cancer Institute's website, press and social media communications and our Program's website, we continually broadly disseminate our vision and results.

What do you plan to do during the next reporting period to accomplish the goals?

During the no cost extension period, we will complete our collection of individual patient AA, white prostate cancer tissue specimens, and patient-matched blood specimens with accompanying annotation and DNA and

RNA isolation. Once complete, we will be performing ancestral genotyping using DNA isolated from the blood specimens and we will be interrogating gene expression, alternative splicing and variation in *cis*-acting splicing elements using DNA and RNA from the tissue specimens. In addition, we will assess the function of RNA splicing regulatory SNPs associated with prostate cancer risk, aggressiveness and survival in driving the RNA splicing mechanism and prostate cancer cell biology. Moreover, we will complete our functional assessment of the AR-V7 and EGFR SSOs. Furthermore, we will test SSOs targeted *PIK3CD* and *MET*. Finally, we will use the AA and white prostate cancer patient-derived primary cell lines and xenografts that we have generated in work that has gone on in parallel with this grant to assess the therapeutic efficacy of our aforementioned SSOs.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our publications related to this project to date have discovered alternative RNA splicing as a novel mechanism promoting tumor aggressiveness and drug resistance in AA prostate cancer. In addition, the associations between RNA splicing regulatory variants of stemness-related genes and racial disparities in susceptibility to prostate cancer identify potential novel biomarkers for racial disparities in prostate cancer risk. Moreover, the single nucleotide polymorphisms of stemness pathway genes predicted to regulate RNA splicing, microRNA and oncogenic signaling that are associated with prostate cancer survival represent potential novel prognostic markers for overall survival of prostate cancer and support a contribution of the stemness pathway to prostate cancer patient outcome.

Our ongoing work to collect individual patient AA and white prostate cancer tissue specimens and patient-matched blood specimens contributes to the goal of accumulating racially diverse prostate tumor models and will contribute to identification of biologically significant factors driving the clinical aggressiveness of AA prostate cancer. In addition, identification of RNA splicing regulatory SNPs that are significantly associated with prostate cancer risk, aggressiveness and/or survival in white and/or AA populations have the potential to serve as novel molecular targets for development of biomarkers of increased risk of aggressive prostate cancer or therapeutics against aggressive prostate cancer. Moreover, development of SSOs to correct aberrant splicing leading to production of AR-V7, to drive production of inhibitory dominant-negative EGFR isoforms and to modulate alternative splicing of *PIK3CD* and *MET* will further our understanding of the contribution of these molecular mechanisms to AA prostate cancer. These SSOs have the potential to yield novel therapeutic modalities to combat prostate cancer in AA men as well as men of all races with aggressive disease driven by these mechanisms.

Finally, in parallel to the work done in the context of this grant, we will make available to other investigators our collection of AA prostate cancer patient-derived primary cell lines and xenografts, after full characterization.

What was the impact on other disciplines?

In parallel to the work done in the context of this grant, we have identified 19 race-related alternative RNA splicing events that also occur in breast, lung and liver cancer. Therefore, the RNA splicing-related concepts and targets and the SSO technology developed here has the potential to have broader applicability.

What was the impact on technology transfer?

1. Along with our qualified collaborator, a US Patent Application has been filed regarding alternative splicing variants of genes associated with prostate cancer risk and survival (US 2014/0364483 A1).
2. Freedman, Jennifer, Patierno, Brendon, LaCroix, Bonnie, Robinson, Timothy, Sullenger, Bruce, George, Daniel and Patierno, Steven. 2015. Splice-switching oligonucleotides and methods of use. 62/250,713, filed November 4, 2015. Patent Pending.
3. Freedman, Jennifer, Wang, Yanru, Liu, Hongliang, Wei, Qingyi, George, Daniel and Patierno, Steven. 2016. Biomarkers for the identification of prostate cancer and methods of use. 15/344,222, filed November 4, 2016. Patent Pending.

What was the impact on society beyond science and technology?

The outreach activities we have undertaken to increase participation, including minority participation, in our GENCADE Study and our physician-patient informed decision-making and informed consent process in our GENCADE Study will improve public knowledge regarding prostate cancer, prostate cancer health disparities among racial groups and clinical research. In collaboration with Duke Cancer Institute's OHED we have helped devise a curriculum to train clinicians and other staff participants in clinical trials on culturally sensitive and competent communications with minority patients regarding clinical trials. In addition, we have improved public knowledge by broadly disseminating our vision and results through community outreach and programming, the Genitourinary Oncology Program at our institution, investors and donors at our institution, the Peter Michael Foundation, the Prostate Cancer Foundation, the American Cancer Society, the American Association for Cancer Research, the American Society of Clinical Oncology, the website at our institution and press and social media communications.

CHANGES/PROBLEMS:**Changes in approach and reasons for change.**

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them.

We have a one-time, extension without funds to the expiration date of our award for a period of 12 months. This extension does not involve a change in our approved objectives or scope of our project. Rather, it enables us to complete our approved interrogation of differentially expressed and spliced candidate genes in AA prostate cancer and determination of the relationship between these genes and Gleason grade. We requested this extension because we have encountered an unanticipated slower rate of accrual collecting individual patient AA and white prostate cancer tissue specimens and patient-matched blood specimens under our IRB approved protocol. Throughout our study, we have dramatically accelerated our rate of collection by optimizing our approach to integrate more seamlessly into clinical operations and our strategy to procure prostate cancer tissue specimens. This extension enables us to complete our collection and the aforementioned analyses using these specimens.

Changes that had a significant impact on expenditures.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to report.

PRODUCTS:**Publications, conference papers, and presentations.**

Peer-reviewed publications:

1. Wang BD, Ceniccola K, Hwang S, Andrawis R, Horvath, A, Freedman JA, Olender, J, Knapp S, Ching T, Garmire L, Patel V, Garcia-Blanco, MA, Patierno SR, Lee NH. Alternative splicing promotes tumour aggressiveness and drug resistance in African American prostate cancer. *Nature Communications*. 2017 Jun 30;8:15921. PMID 28665395.
2. Wang Y*, J.A. Freedman*, X. Li, P.G. Moorman, D.J. George, T. Hyslop, S.R. Patierno** and Q. Wei**. Associations between RNA splicing regulatory variants of stemness-related genes and racial disparities in susceptibility to prostate cancer. *International Journal of Cancer*. 2017;141(4):731-743. PMID: 28510291. *Contributed equally as co-first authors, ** Contributed equally as co-PIs.
3. Freedman JA*, Wang Y*, Li X, Moorman PG, George DJ, Hyslop T, Wei Q**, Patierno SR**, Single nucleotide polymorphisms of stemness pathway genes predicted to regulate RNA splicing, microRNA

and oncogenic signaling are associated with prostate cancer survival, manuscript in preparation,
 *Contributed equally as co-first authors, **Contributed equally as co-PIs.

Conference presentations:

1. LaCroix, B., B. M. Patierno, B. A. Sullenger, D. J. George, S. R. Patierno and J. A. Freedman. Development of a novel therapeutic splice-switching oligonucleotide targeting race-related androgen receptor signaling and aggressive prostate cancer. AACR Conference on The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved. Atlanta, Georgia. 2017. Oral presentation.
2. LaCroix, B., B. M. Patierno, B. A. Sullenger, D. J. George, S. R. Patierno and J. A. Freedman. Development of a novel therapeutic splice-switching oligonucleotide targeting race-related androgen receptor signaling and aggressive prostate cancer. AACR Conference on The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved. Atlanta, Georgia. 2017. Poster presentation.
3. Deveaux, A., B-D. Wang, B. LaCroix, B. M. Patierno, D. Zhang, K. Owzar, N. H. Lee, D. J. George, J. A. Freedman and S. R. Patierno. Race-related differential splicing of the insulin receptor: A novel target underlying prostate cancer disparities. AACR Conference on The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved. Atlanta, Georgia. 2017. Poster presentation.
4. Patierno, B. M., W. Glover, T. Ribar, R. A. Kittles, W-C. Foo, S. J. McCall, J. Huang, D. J. George, J. A. Freedman, S. R. Patierno, K. Wood and D. S. Hsu. Establishment of African American prostate cancer patient-derived primary cell lines and xenografts. AACR Conference on The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved. Atlanta, Georgia. 2017. Poster presentation.
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6. Freedman, J. A.^{*}, Y. Wang^{*}, H. Liu, A. Deveaux, P. G. Moorman, T. Hyslop, D. J. George, N. H. Lee, Q. Wei^{**} and S. R. Patierno^{**}. Single nucleotide polymorphisms of race-related alternatively spliced genes associate with prostate cancer risk, aggressiveness, and/or survival. Duke Cancer Institute Scientific Retreat. Durham, North Carolina. 2016. Oral and Poster Presentation. ^{*}Contributed equally as co-first authors, ^{**}Contributed equally as co-PIs.
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cancer in men of African descent. Poster Presentation. Duke Cancer Institute Scientific Retreat. Durham, NC. 2015.

Website(s) or other Internet site(s).

Nothing to report.

Technologies or techniques.

We have developed SSOs to correct aberrant splicing leading to production of AR-V7, to drive production of inhibitory dominant-negative EGFR isoforms and to manipulate *PIK3CD* and *MET* alternative splicing and to drive production of inhibitory EGFR isoforms and, once complete, we plan to submit the research data for publication making our scientific discovery open to the scientific community. In addition, in parallel to the work being done in the context of this grant, we have established a number of AA and white prostate cancer patient-derived primary cell lines and xenografts. After characterization, we plan to submit the research data for publication making our scientific discovery open to the scientific community and will provide our models to the community.

Inventions, patent applications, and/or licenses.

1. Along with our qualified collaborator, a US Patent Application has been filed regarding alternative splicing variants of genes associated with prostate cancer risk and survival (US 2014/0364483 A1).
2. Freedman, Jennifer, Patierno, Brendon, LaCroix, Bonnie, Robinson, Timothy, Sullenger, Bruce, George, Daniel and Patierno, Steven. 2015. Splice-switching oligonucleotides and methods of use. 62/250,713, filed November 4, 2015. Patent Pending.
3. Freedman, Jennifer, Wang, Yanru, Liu, Hongliang, Wei, Qingyi, George, Daniel and Patierno, Steven. 2016. Biomarkers for the identification of prostate cancer and methods of use. 15/344,222, filed November 4, 2016. Patent Pending.

Other products.

Nothing to report.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	Steven Patierno, PhD, PI
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	Provided senior level oversight and direction of research, oversaw the GENCADE study
Funding Support	P30CA014236-42 See attached other support/ DOD

Name:	Daniel J George, MD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	Oversaw the GENCADE study, contributed to oversight and direction of research
Funding Support	See attached other support/ DOD

Name:	Jennifer A Freedman, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.1
Contribution to Project:	Oversaw collection of GENCADE specimens in collaboration with the Genitourinary Oncology clinical research team, identified variation in cis-acting splicing regulatory sequences of race-related alternatively spliced genes that associates with prostate cancer risk, aggressiveness and survival, assessed function of splice-switching oligonucleotides targeting AR-V7 and EGFR, designed splice-switching oligonucleotides to manipulate PIK3CD and MET alternative splicing, managed and mentored Research Analyst II, Bonnie LaCroix, oversaw establishment of new models of African American prostate cancer
Funding Support	See attached other support/ DOD

Name:	Yuan Wu
Project Role:	Biostatistician
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Contribution to Project:	Statistical plan for GENCADE study
Funding Support	

Name:	Norman Lee, PhD
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Nearest person month worked:	1.2
Contribution to Project:	Coordinated efforts between GWU and Duke, oversaw GENCADE study at GWU, tested splice-switching oligonucleotides targeting PIK3CD
Funding Support	See attached other support/ DOD

Name:	Ramez Andrawls, MD
Project Role:	GWU Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	Collected GENCADE specimens at GWU
Funding Support	See attached other support/ DOD

Collaborating Organization – George Washington University Medical Center

ABSTRACT (approximately 200 words):

We have discovered RNA splicing as a novel mechanism underlying tumor aggressiveness and drug resistance in African American (AA) prostate cancer (PCa). To interrogate further the contribution of RNA splicing to the more aggressive PCa biology in AA men, we are collecting AA and white PCa patient blood and tissue specimens of varying Gleason grade for study. In addition, we have identified RNA splicing regulatory variants that associate with PCa risk, aggressiveness and/or survival. Furthermore, we have developed a splice-switching oligonucleotide (SSO) that inhibits production of a pathogenic androgen receptor (AR) variant at the RNA- and protein-level, while maintaining expression of full-length AR, which has therapeutic value. This SSO inhibits proliferation of PCa cells and restores sensitivity to an AR inhibitor. In addition, we have developed SSOs that drive production of inhibitory dominant-negative epidermal growth factor receptor (EGFR) isoforms at the RNA-level and decrease phosphorylated EGFR protein. Ultimately, this study will establish novel biological differences between AA and white PCa and their relevance to tumor biology, which will aid in the development of new biomarkers and/or therapeutics that will reduce PCa health disparities for AAs and improve outcomes for men of all races with aggressive disease.

Figure 1. A. Overall flowchart. B. Kaplan-Meier survival curves of prostate cancer patients according to combined risk alleles of the five independent and significant SNPs in low-, median-, and high-risk groups. Abbreviations: PLCO: The Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; SNP: Single nucleotide polymorphism; FPRP: False positive report probability; MAF: Minor allele frequency; HWE: Hardy-Weinberg equilibrium; ROC: Receiver operating characteristic.

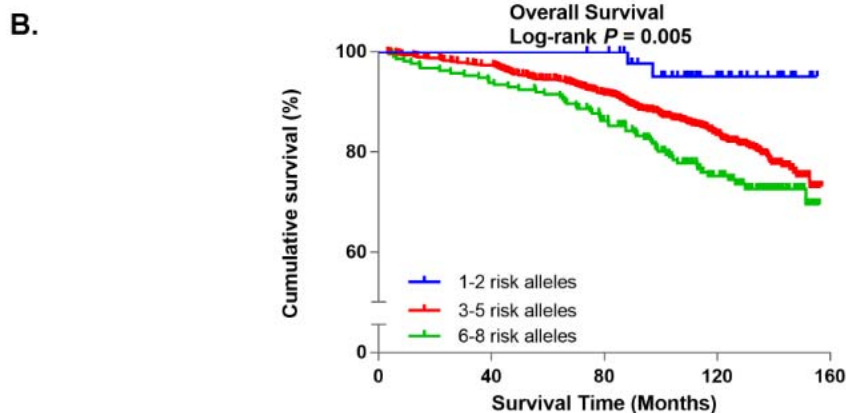
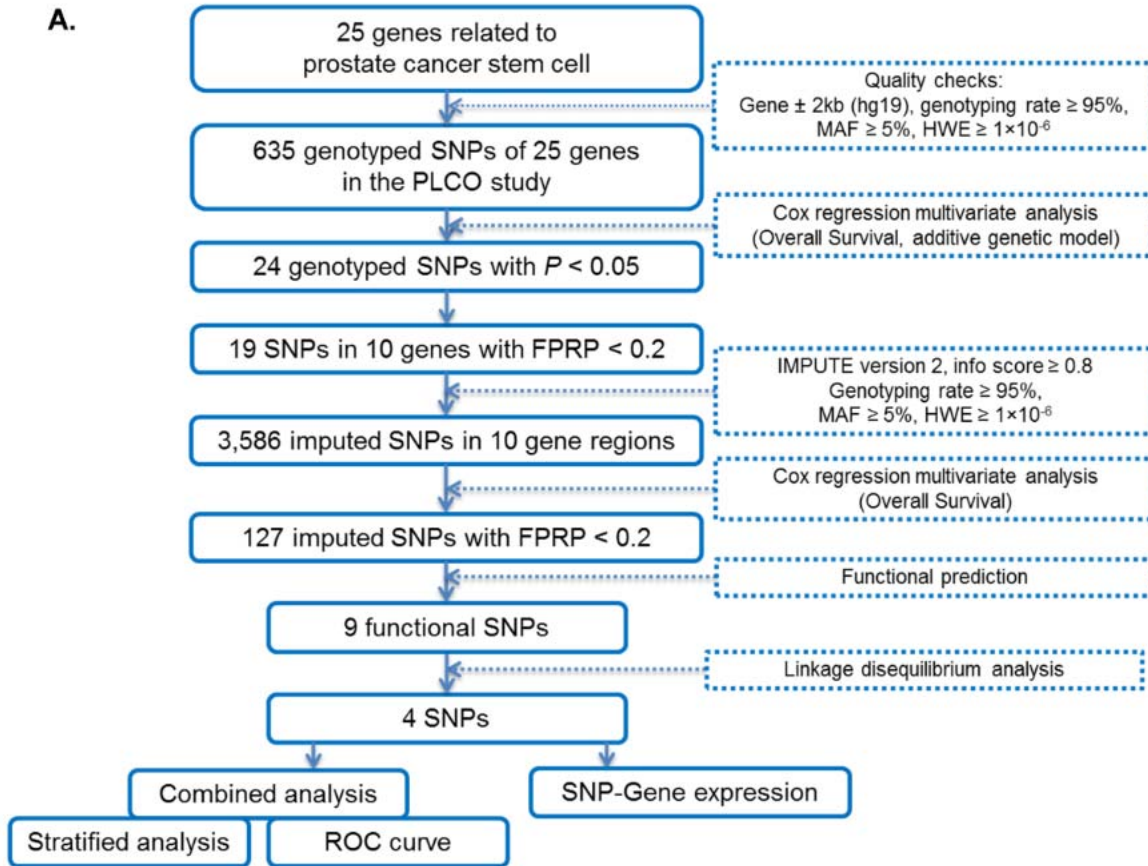


Figure 2. Receiver-operating characteristic (ROC) curves for prediction of prostate cancer survival based on only clinical characteristics (age, Gleason score and tumor stage) or additionally combined effect genotypes, (A) time-dependent area under curve (AUC) and (B) ROC curves corresponding to the time point of ten-years.

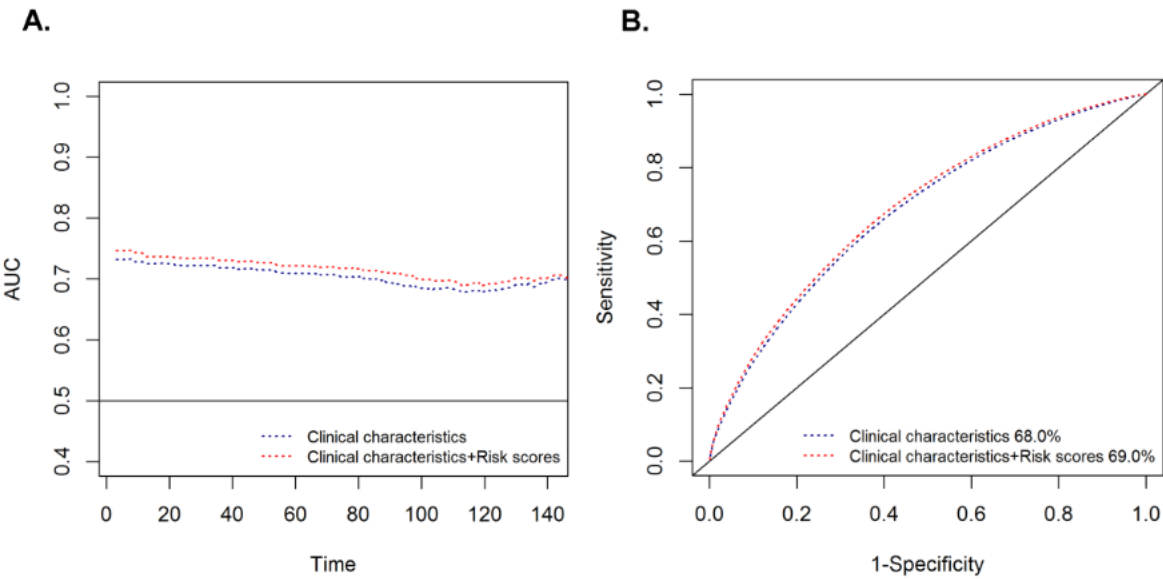


Figure 3. Correlations between the four independent and significant SNPs and the relative mRNA expression levels in lymphoblastoid cell lines of 716 individuals from HapMap 3 Project including 107 CEU, 242 CHB, 41 MEX and 326 AFR populations. A, C, E and G are in all populations with 716 individuals. B, D, F and H are only in 107 CEU individuals. (A, B) rs9666607 and *CD44* (reporter: ILMN_1732193); (C, D) rs35605 and *ABCC1* (reporter: ILMN_1802404); (E, F) rs212091 and *ABCC1* (reporter: ILMN_1802404); (G, H) rs1058587 and *GDF15* (reporter: ILMN_1763658). P_{adj} is from linear regression analysis adjusted for age and/or ethnic groups.

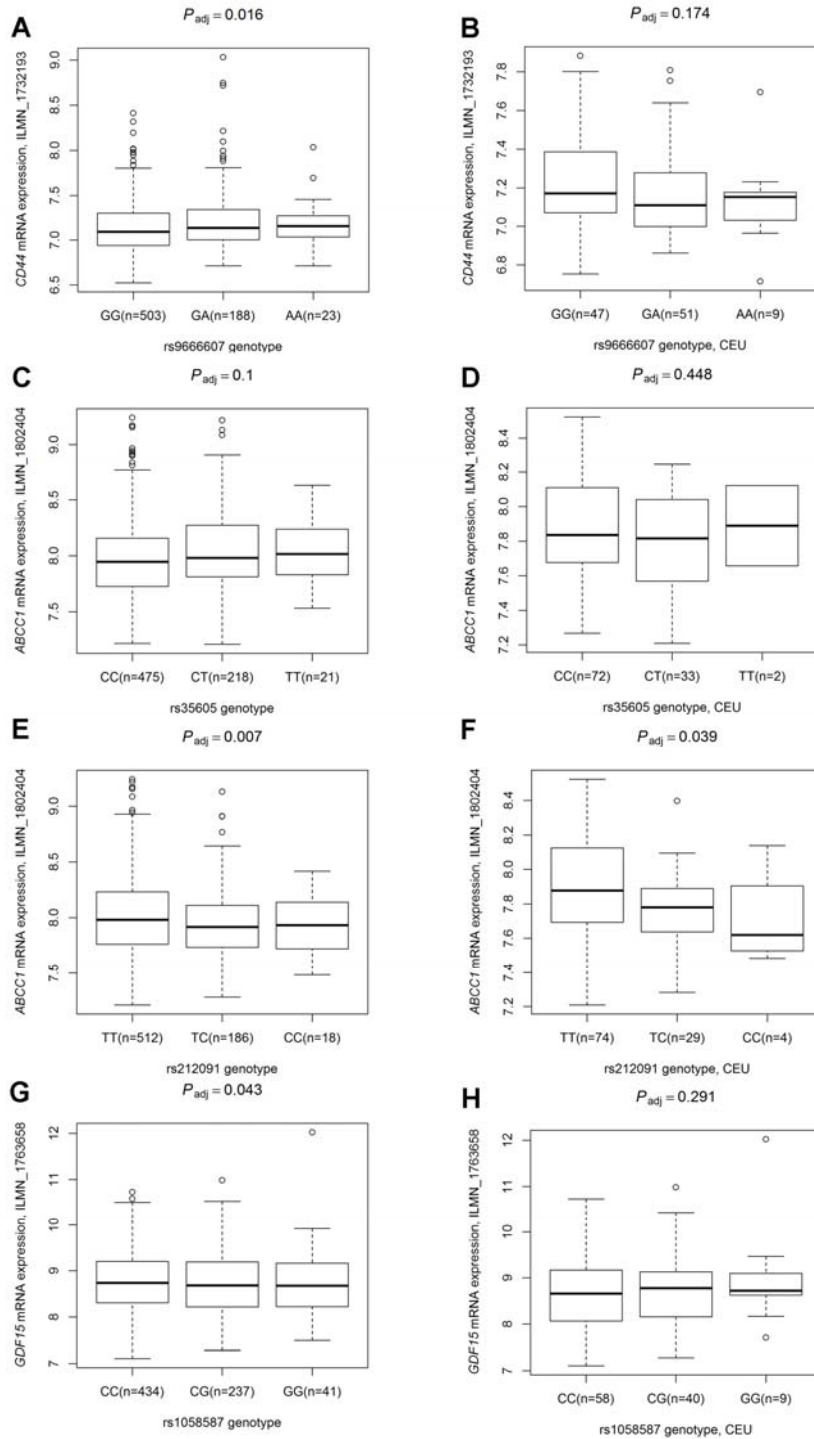
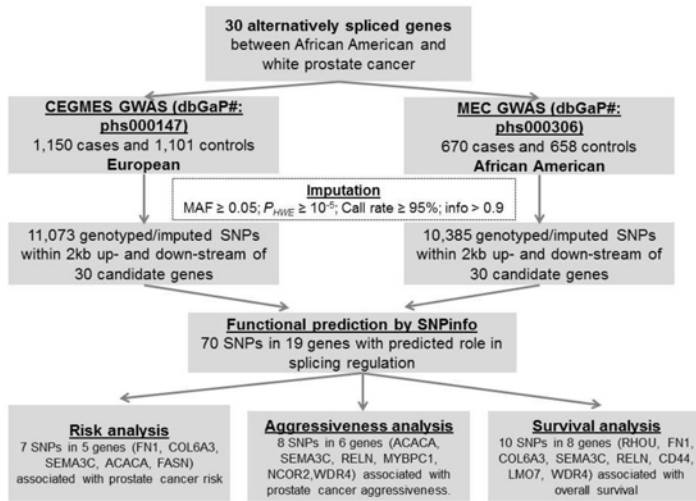


Figure 4. A. Study workflow. B. Associations between SNPs of race-related alternatively spliced genes in prostate cancer and prostate cancer risk (top), aggressiveness (middle) and survival (bottom). All SNPs predicted to play a role in splicing regulation.

A.



B.

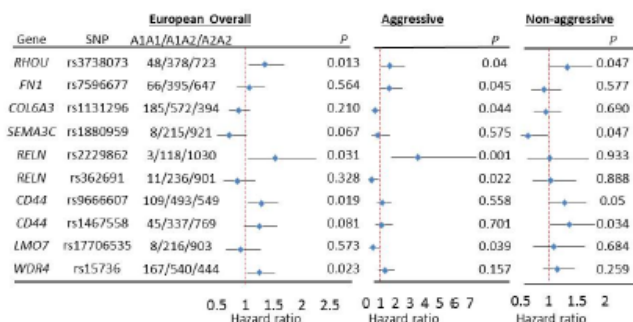
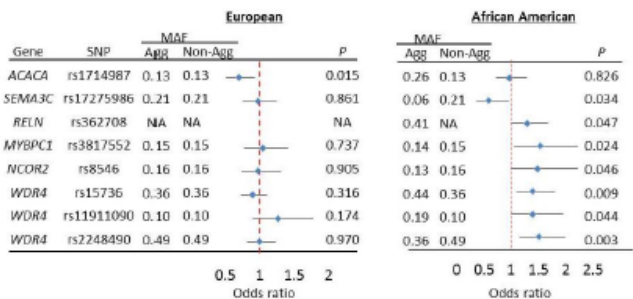
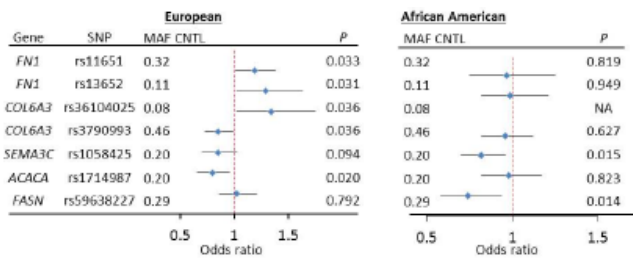


Figure 5. A and B. Dose-dependent inhibition of AR-V7 RNA and protein, respectively, by the AR-V7 SSO. C and D. Maintenance of expression of full-length AR RNA and protein, respectively, post-treatment with the AR-V7 SSO. E. Inhibition of prostate cancer cell proliferation and restoration of sensitivity to the AR inhibitor enzalutamide, respectively, by the AR-V7 SSO. W, white. AA, African American. Scramble/Scr, control scrambled oligonucleotide.

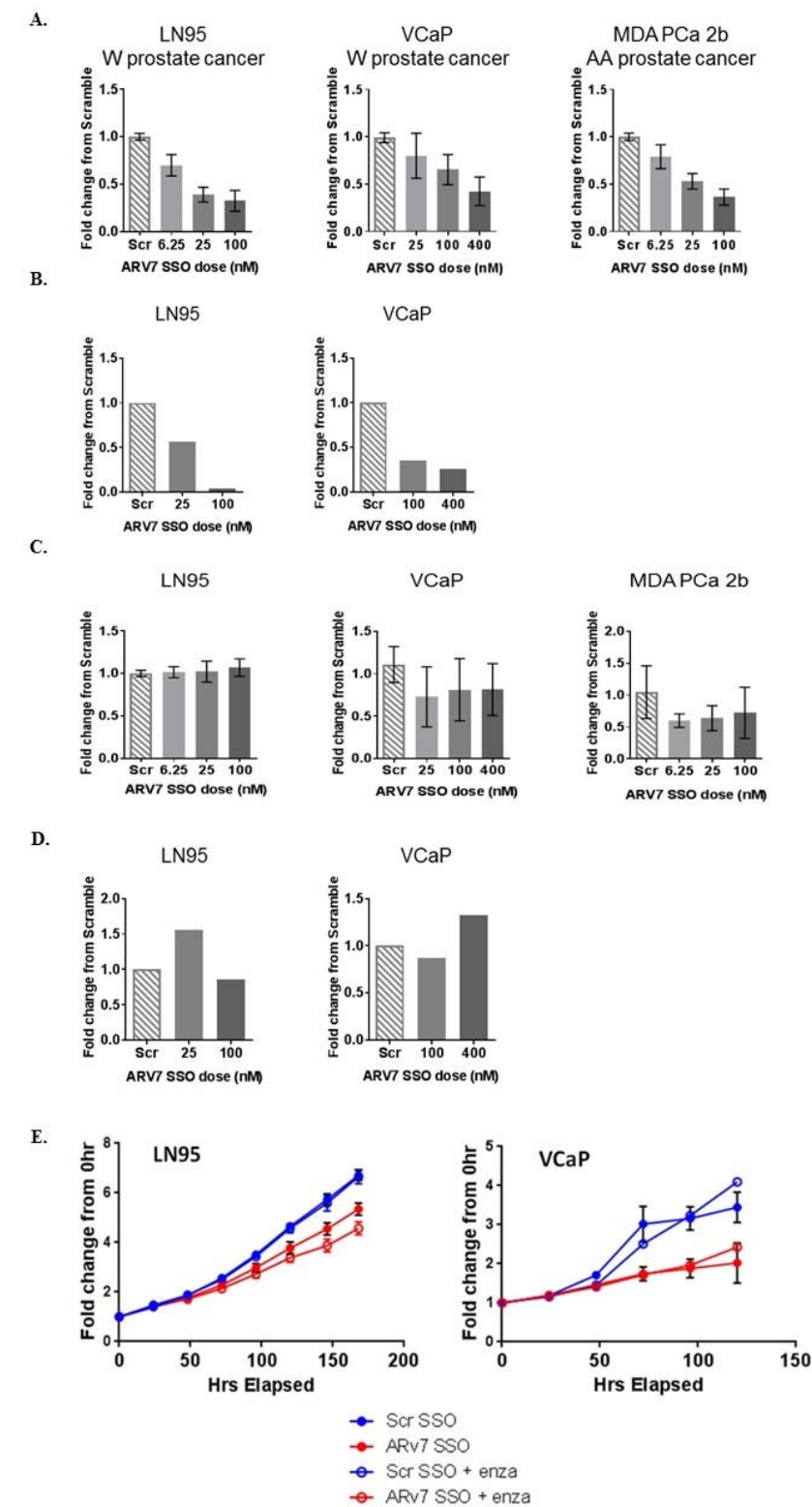


Figure 6. EGFR-TM (Ex16) and –TK (Ex18) SSOs drive production of dominant-negative EGFR variants in a dose-dependent manner (left) and inhibit pEGFR expression (right) in LNCaP prostate cancer cells. NTC, no treatment control.

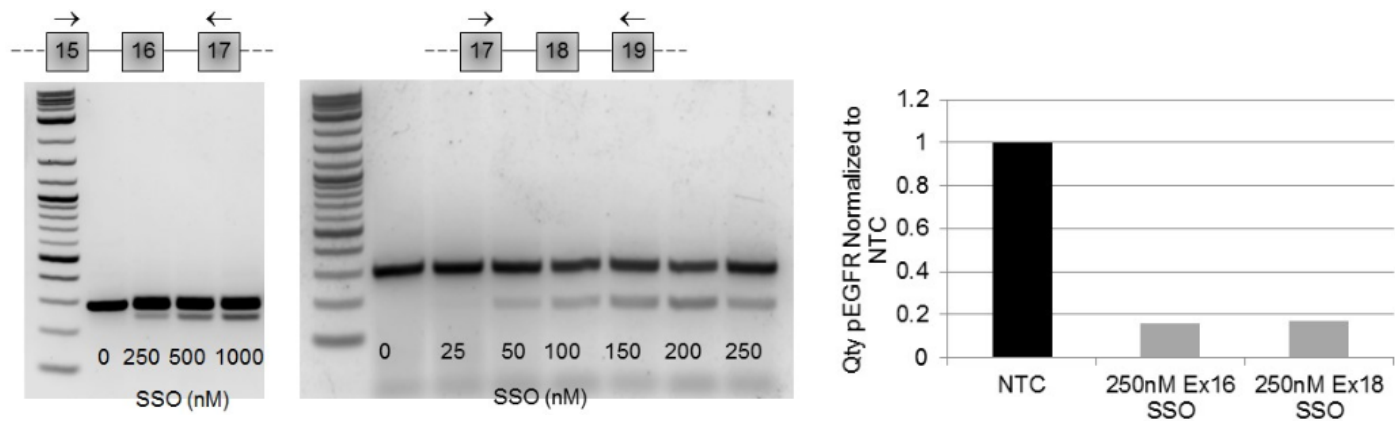


Figure 7. Representative examples of AA prostate cancer patient-derived A. primary cell lines and B. xenografts being generated.

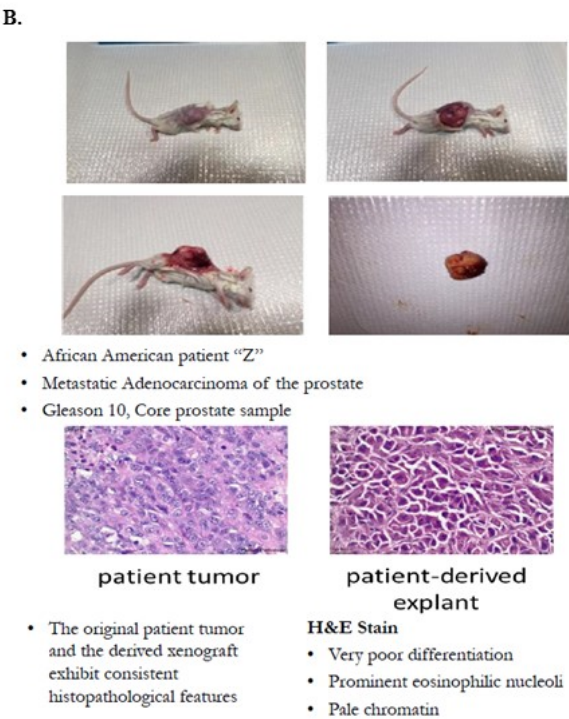
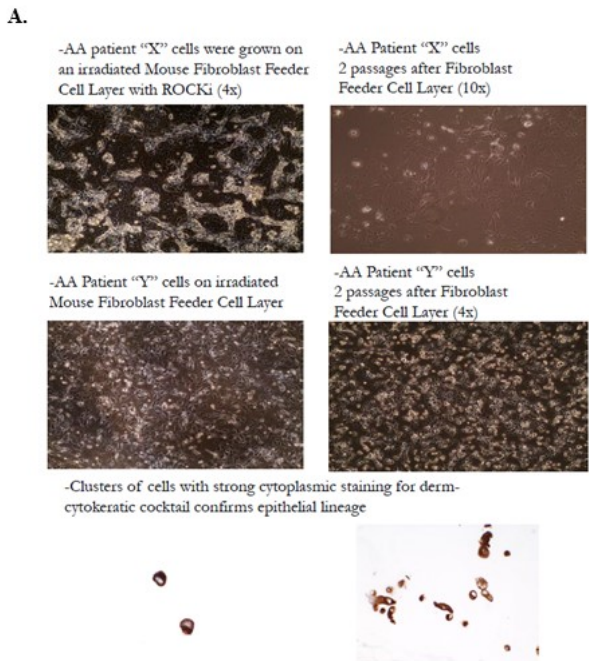


Table 1: Clinical characteristics of the prostate cancer PLCO study in overall survival.

Characteristics	Frequency		Univariate Analysis		Multivariate Analysis ^a	
	All	Deaths (%)	HR (95% CI)	P	HR (95% CI)	P
Overall	1150	215 (18.7)				
Age (years)						
Median (range)	67 (55-81)					
< 67	544	70 (12.9)	1.00		1.00	
≥ 67	606	145 (23.9)	2.24 (1.68-2.98)	< 0.001	1.80 (1.34-2.42)	< 0.001
PSA before diagnosis (ng/ml)						
Median (range)	6.1 (0.05-1137)					
< 6.1	572	89 (15.6)	1.00		1.00	
≥ 6.1	578	126 (21.8)	1.38 (1.05-1.81)	0.021	0.98 (0.74-1.30)	0.887
Gleason score						
2≤&≤ 6	567	96 (16.9)	1.00		1.00	
= 7	464	76 (16.4)	1.19 (0.88-1.61)	0.254	1.23 (0.90-1.70)	0.199
≥ 8	114	41 (36.0)	3.13 (2.17-4.53)	< 0.001	2.52 (1.68-3.77)	< 0.001
Missing	5					
Stage						
I/II	913	153 (16.7)	1.00		1.00	
III/IV	237	62 (26.2)	1.62 (1.20-2.17)	0.002	1.76 (1.26-2.46)	0.001
Aggressiveness ^b						
Non-aggressive	489	78 (16.0)	1.00		1.00	
Aggressive	659	137 (20.8)	1.65 (1.24-2.18)	0.001	1.89 (1.40-2.56)	< 0.001
Missing	2					
Types of treatments						
Radical prostatectomy	614	75 (12.2)	1.00		1.00	
Radiotherapy alone	194	46 (23.7)	2.00 (1.39-2.89)	< 0.001	2.06 (1.39-3.06)	< 0.001
Radiotherapy + Endocrine therapy	202	40 (19.8)	1.96 (1.34-2.88)	0.001	1.56 (1.03-2.36)	0.035
Endocrine therapy alone	54	29 (53.7)	7.21 (4.68-11.09)	< 0.001	4.28 (2.64-6.92)	< 0.001
Other treatments	86	25 (29.1)	2.53 (1.61-3.98)	< 0.001	2.68 (1.65-4.36)	< 0.001

Abbreviations: PLCO: The Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; HR: Hazards ratio; CI: Confidence interval; PSA: Prostate specific antigen; The median follow-up time is 121.7 months.

^a Multivariate cox regression analyses were adjusted for age, Gleason score, PSA level, stage and primary treatments. In subgroup of "Aggressiveness", we included age, PSA level, and primary treatment for adjustments.

^b Non-aggressive: cases with a Gleason score < 7 and stage < III; Aggressive: cases with a Gleason score ≥ 7 or stage ≥ III.

Table 2: The four independent and significant SNPs with potential functions.

SNP	Gene	Chr.	Position	Location	Allele ^a	EAF	SNPinfo ^b	RegulomeDB ^c	HR (95% CI) ^d	P ^d	FPRP ^d
rs9666607	<i>CD44</i>	11	35226155	Exon	G/A	0.31	Splicing	5	1.28 (1.04-1.58)	0.018	0.141
rs35605	<i>ABCC1</i>	16	16162019	Exon	C/T	0.16	Splicing	1f	0.71 (0.53-0.94)	0.018	0.138
rs212091	<i>ABCC1</i>	16	16236650	3'UTR	T/C	0.15	miRNA	7	0.58 (0.43-0.80)	0.001	0.009
rs1058587	<i>GDF15</i>	19	18499422	Exon	C/G	0.26	nsSNP	4	1.29 (1.05-1.59)	0.015	0.117

Abbreviations: SNP: Single nucleotide polymorphism; nsSNP: Non-synonymous SNP; Chr.: Chromosome; EAF: Effect allele frequency; HR: Hazards ratio; CI: Confidence interval; UTR: Untranslated region; FPRP: False positive report probability.

^aReference/effect allele.

^bSNPinfo, <http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm>.

^cRegulomeDB, <http://regulome.stanford.edu/>, SNPs with predicted scores of "1" were considered as functional.

^dMultivariate cox regression analyses were adjusted for age, Gleason score, stage and primary treatments.

Table 3: Combined analysis of the four independent and significant SNPs in four risk groups.

Number of risk alleles ^a	Frequency		Multivariate analysis ^b		AIC ^c
	All	Deaths (%)	HR (95% CI)	P	
1-2	46	2 (4.3) ^d	1.00		
3	158	19 (12.0)	4.81 (1.10-21.00)	0.037	
4-5	709	138 (19.5)	8.35 (2.04-34.19)	0.003	
6-8	213	53 (24.9)	11.80 (2.34-49.04)	0.001	
Trend				5.30E-07	2666.68

Abbreviations: SNP: Single nucleotide polymorphism; HR: Hazards ratio; CI: Confidence interval; AIC, Akaike information criterion.

^aRisk alleles were rs9666607 A, rs35605 C, rs212091 T and rs1058587 G.

^bMultivariate cox regression analyses were adjusted for age, Gleason score, stage, and primary treatments.

^cAIC in the trend model of multivariate cox regression analyses.

^dTwo patients reached the endpoint were both with two risk alleles.